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14. ABSTRACT

We find that the ErbB2 receptor tyrosine kinase is active in vestibular schwannoma (VS) cells and drives proliferation. Our overall hypothesis is that defects in merlin lead to constitutive ErbB2 activation at the cell membrane and that inhibition of ErbB2 will reduce the survival of VS cells and potentiate the effects of radiation on VSs. In this report we show that VS cells, which lack functional merlin, constitutively express activated ErbB2 in lipid rafts contributing to their proliferative potential. Furthermore, protein kinase A inactivates merlin by phosphorylation in Schwann cells in vitro and in vivo following denervation, correlated with movement of ErbB2 into lipids rafts and re-entry into the cell cycle. VS cells are relatively radioresistance, due at least in part, to persistent JNK activity which promotes cell survival and limits oxidative stress. Finally, inhibition of ErbB2 reduces VS cell radiosensitivity whereas activation of ErbB2 enhances radiosensitivity, likely by regulating cell proliferation.

15. SUBJECT TERMS
Vestibular schwannoma, neurofibromatosis, radiation, merlin, ErbB2

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Table of Contents

Introduction:

Neurofibromatosis type II (NF2) results from mutation in the tumor suppressor gene, *merlin*, leading to the development of multiple intracranial and spinal tumors including schwannomas. Schwann cells (SCs) of the vestibular nerve are most commonly affected and bilateral vestibular schwannomas (VSs) is a hallmark of NF2. We have begun exploring factors that contribute to VS growth. We find that the ErbB2 receptor tyrosine kinases is active in VS cells and drives proliferation. *Our overall hypothesis is that defects in merlin lead to constitutive ErbB2 activation at the cell membrane and that inhibition of ErbB2 will reduce the survival of VS cells and potentiate the effects of radiation on VSs*. Our object is to test this hypothesis using cultured primary human VS cells.

Body:

During the second year of the award we have made substantial progress in achieving the aims of the proposal. Below we discuss the progress for each aim of the proposal.

Specific aim 1. Determine the ability of merlin to regulate ErbB2 localization and activity in vestibular schwannoma (VS) cells. We initially focused on correlating the status of merlin

phosphorylation with ErbB2 trafficking in normal Schwann cells (SCs). First, we determined that the trafficking of ErbB2 into lipid rafts in SCs correlates with loss of axonal contact, phosphorylation of merlin on Serine 518, and proliferation (Figs. 1 and 2). Thus, phosphorylation of merlin on Serine 518 (S518), which inhibits its growth suppressive function, is correlated with the movement of ErbB2 into lipid rafts in the cell membrane. We also found that ErbB2 constitutively resides in lipid rafts in human VS cells that lack functional merlin.[1] In the coming year we will use cultured VS cells to determine the extent to which replacement of merlin in human vestibular schwannoma cells regulates the trafficking of ErbB2 within the cell membrane.

Figure 1. **A**. Immunoblots of contralateral nerve (**CL**), proximal (**P**) and distal (**D**) segments of the rat sciatic nerve 4 and 7 days post-axotomy (**PA**) probed with anti-merlin S518 phosphorylation (**p-merlin**) and non-phosphospecific merlin antibodies. **B**. Immunostaining of frozen sections from contralateral and distal nerve segments with anti-pmerlin (green) and anti-neurofilament 200 (NF200, red) antibodies.

Specific aim 2. Determine whether phosphorylation of merlin on serine 518 (S518) by protein kinase A (PKA) inhibits merlin's ability to regulate ErbB2 trafficking and suppress VS cell proliferation. We first demonstrated that activation of protein kinase A (PKA) with forskolin (FSK 5 μM) leads to merlin S518 phosphorylation in rat SCs (Fig. 3) and that this correlates with proliferation and with movement of ErbB2 to the cell membrane (see above). Furthermore, we found that treatment of sectioned sciatic nerves with the PKA inhibitor, KT5720, reduces merlin phosphorylation following axotomy, verifying that PKA phosphorylates merlin in proliferating SCs *in vivo*. In the coming year, we will return to cultured VS cells to directly test the role of S518 phosphorylation by PKA in regulating ErbB2 trafficking and VS proliferation.
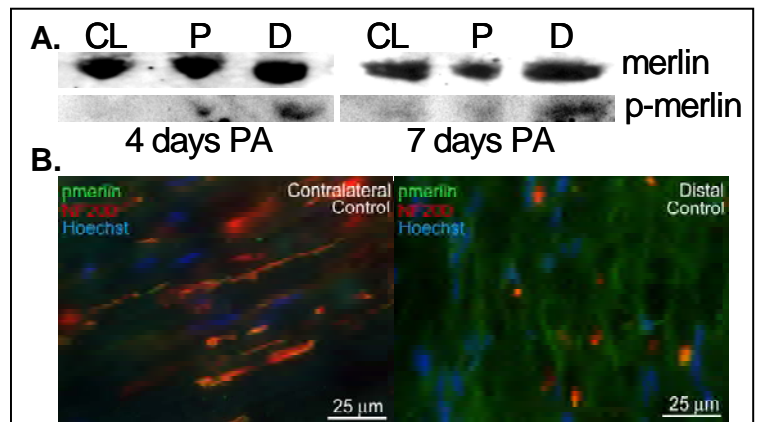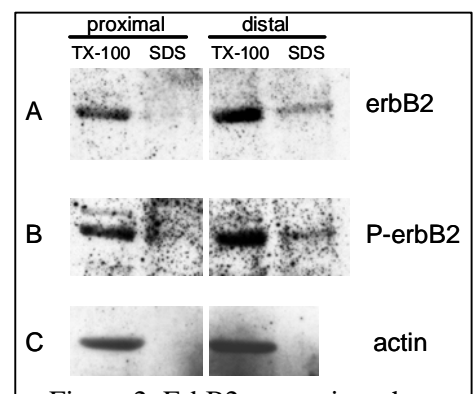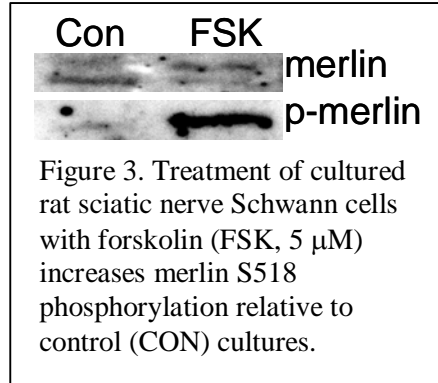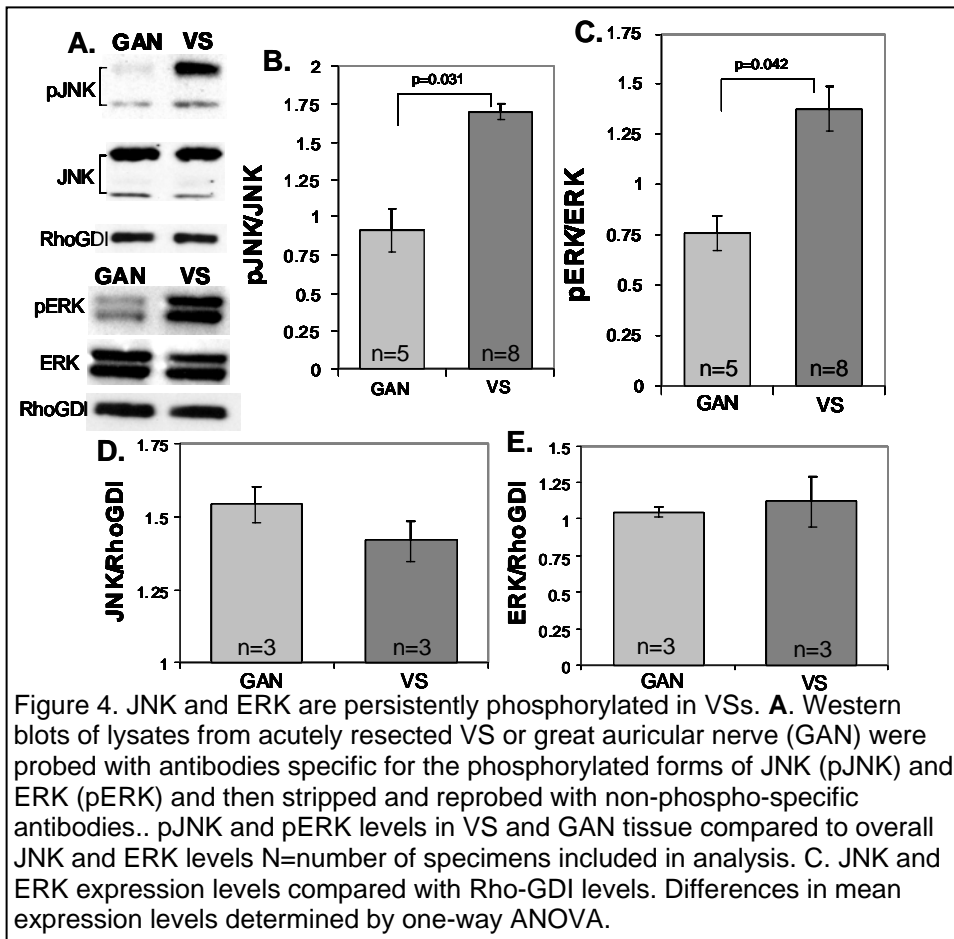

Figure 2. ErbB2 moves into the Triton-X-100 insoluble (lipid raft) fraction and becomes phosphorylated (p-ErbB2) in denervated Schwann cells (distal) following axotomy.

2

Specific aim 3. Determine whether ErbB2 inhibitors potentiate the ability of radiation therapy (RT) to induce VS apoptosis and reduce proliferation. We have been able to perform radiation experiments on several cultured human VS specimens. We demonstrate that doses of ≥30 Gy are required to limit VS cell proliferation and that doses ≥40 Gy are required to induce apoptosis. We found that sublethal doses of radiation (10 Gy) induce DNA damage in VS cells, evidenced by histone 2AX phosphorylation, implying that VS cells possess intrinsic mechanisms to repair radiation induced DNA damage without undergoing apoptosis. Furthermore, we show that inhibition of ErbB2, which reduces VS cell proliferation, protects VS cells against radiation induced cell death. Conversely, activation of ErbB2 by treatment with neuregulin, which promotes VS cell proliferation, increases the radiosensitivity of human VS cells. These results imply that (1) VS are radioresistant relative to most neoplasms, (2) the radiosensitivity of human VS cells depends on proliferative status, and (3) ErbB2 signaling sensitizes VS cells to radiation by promoting proliferation. This work is now published.[2] Thus, we have been able to make substantial progress on the proposed experiments in this aim. To extend these observations, we have examined the effects of merlin-sensitive downstream kinases, MEK, Akt, and c-Jun N-terminal kinase (JNK) on VS cell proliferation, apoptosis and radiosensitivity. We find that JNK is active in VS cells and promotes cell proliferation and survival by limiting the accumulation of reactive oxygen species (Figs. 4-8). Further, JNK confers a radioprotective effect on VS cells evidenced by increased apoptosis in VS cultures treated concurrently with γ-irradiation and JNK inhibitors, SP600125 or I-JIP (Fig. 9). Finally, we have found that ErbB2 inhibitors reduce the growth of VS xenografts in nude mice, demonstrating the therapeutic relevance and potential of these investigations.[3]
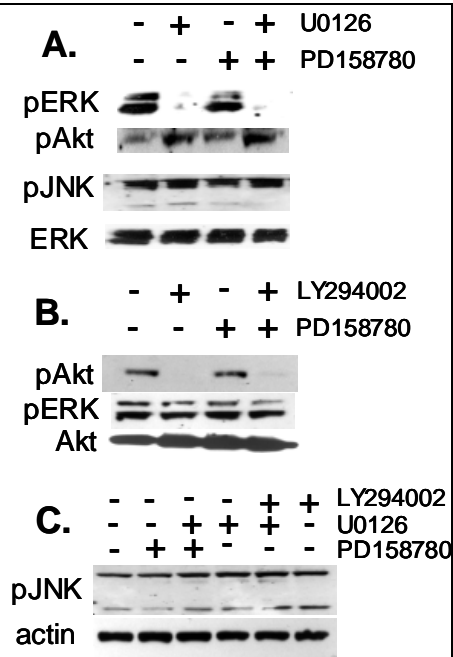

Figure 3. Treatment of cultured rat sciatic nerve Schwann cells with forskolin (FSK, 5 μM) increases merlin S518 phosphorylation relative to control (CON) cultures.

Figure 4. JNK and ERK are persistently phosphorylated in VSs. **A**. Western blots of lysates from acutely resected VS or great auricular nerve (GAN) were probed with antibodies specific for the phosphorylated forms of JNK (pJNK) and ERK (pERK) and then stripped and reprobed with non-phospho-specific antibodies.. pJNK and pERK levels in VS and GAN tissue compared to overall JNK and ERK levels N=number of specimens included in analysis. C. JNK and ERK expression levels compared with Rho-GDI levels. Differences in mean expression levels determined by one-way ANOVA.
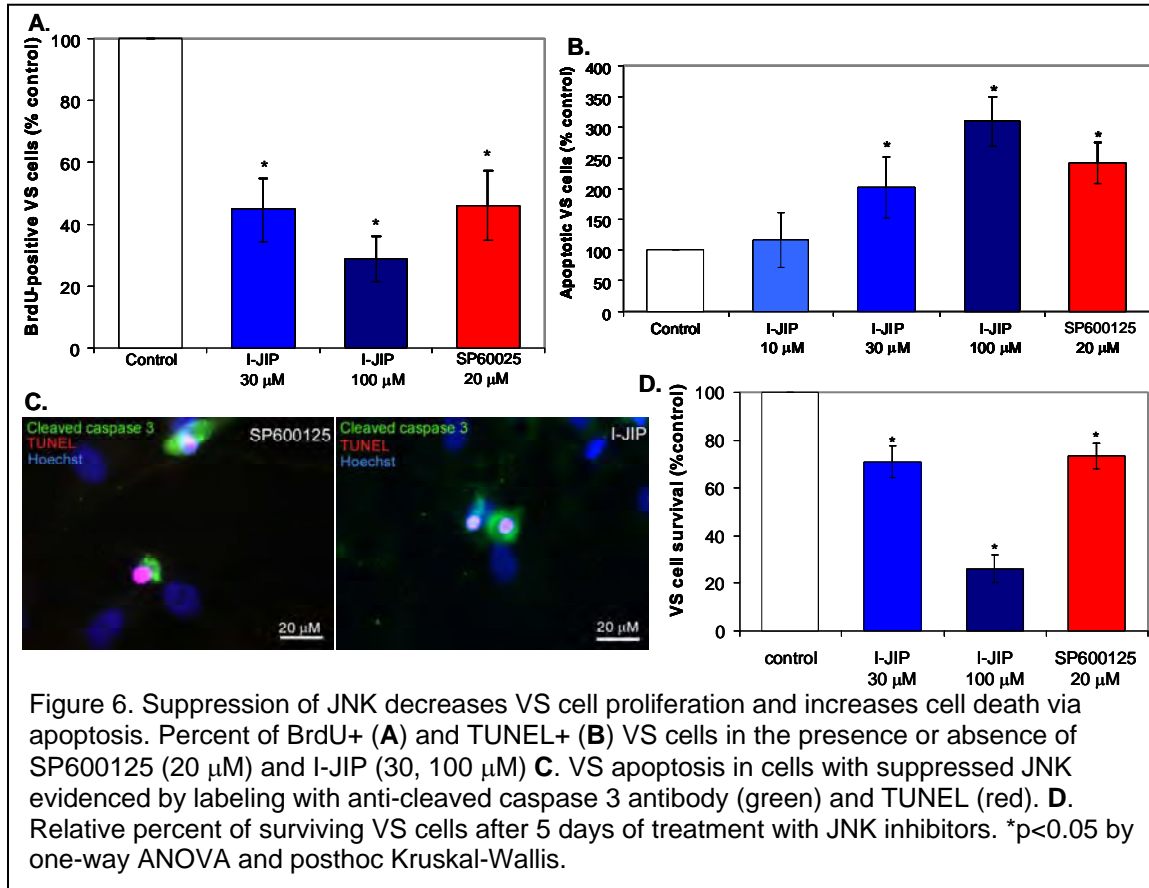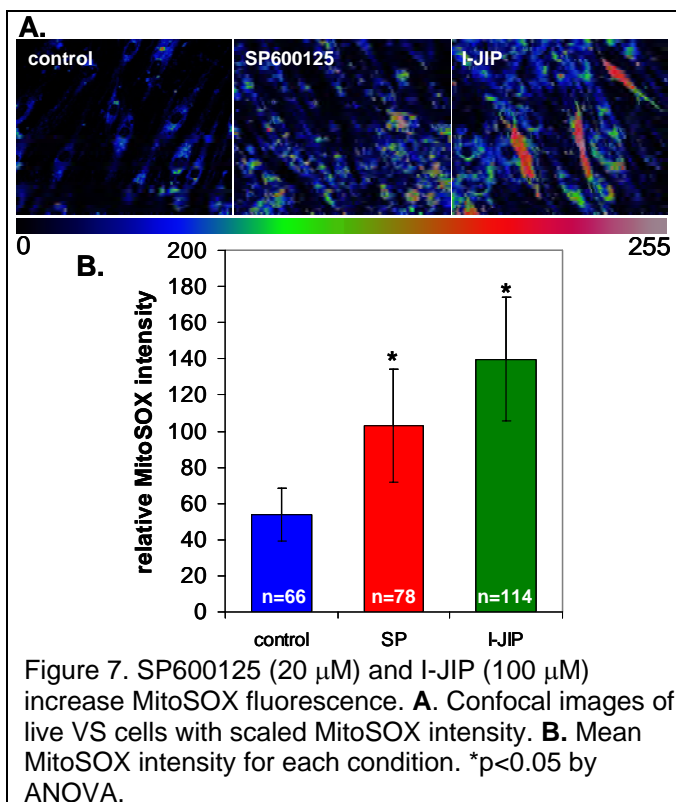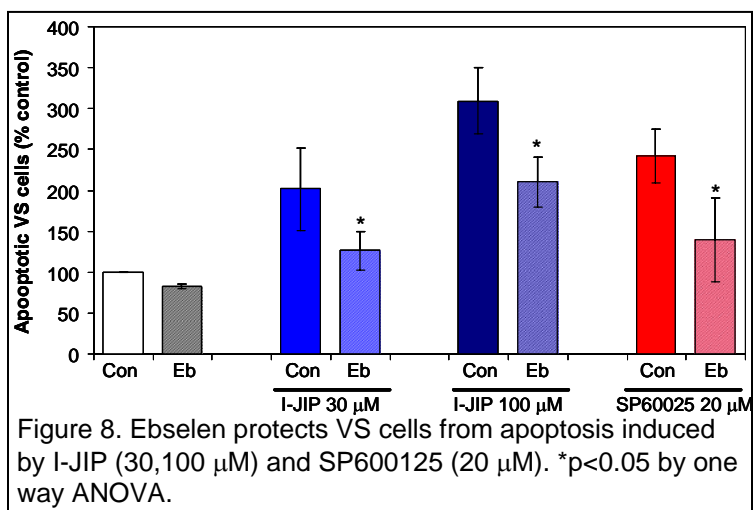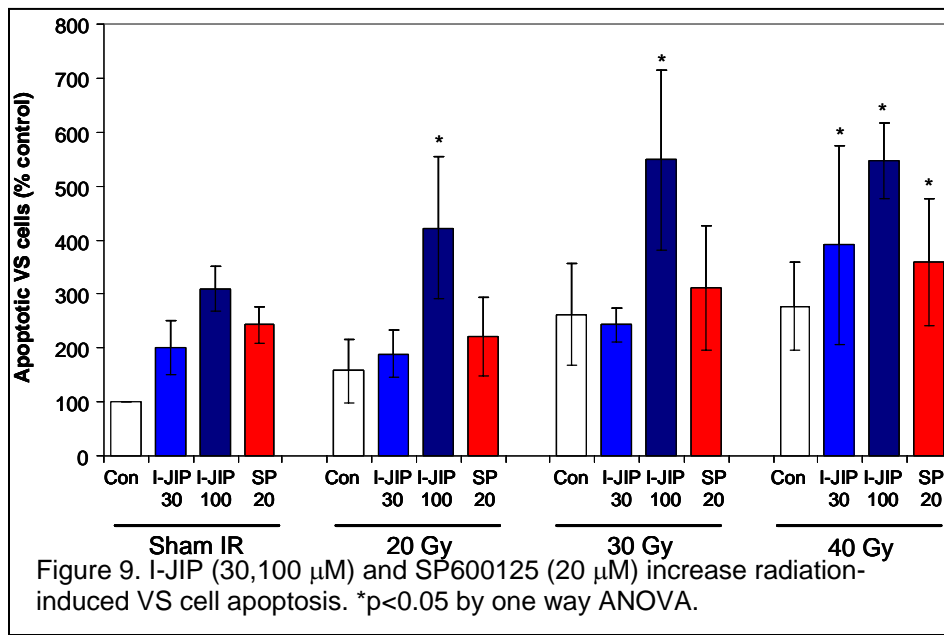
4

Figure 5. JNK, ERK, and Akt are persistently phosphorylated in cultured VS cells. Western blots of lysates from VS cultures maintained in the presence or absence of the indicated inhibitors were probed with antibodies specific for the phosphorylated forms of ERK (pERK), AKT (pAKT), or JNK (pJNK). The ErbB2 inhibitor, PD158780, did not reduce pERK, pAkt, nor pJNK levels. Similar results were obtained in 3 separate VS cultures.

Figure 6. Suppression of JNK decreases VS cell proliferation and increases cell death via apoptosis. Percent of BrdU+ (**A**) and TUNEL+ (**B**) VS cells in the presence or absence of SP600125 (20 μM) and I-JIP (30, 100 μM) **C**. VS apoptosis in cells with suppressed JNK evidenced by labeling with anti-cleaved caspase 3 antibody (green) and TUNEL (red). **D**. Relative percent of surviving VS cells after 5 days of treatment with JNK inhibitors. *p<0.05 by one-way ANOVA and posthoc Kruskal-Wallis.

Figure 7. SP600125 (20 μM) and I-JIP (100 μM) increase MitoSOX fluorescence. **A**. Confocal images of live VS cells with scaled MitoSOX intensity. **B.** Mean MitoSOX intensity for each condition. *p<0.05 by ANOVA.

Figure 8. Ebselen protects VS cells from apoptosis induced by I-JIP (30,100 μM) and SP600125 (20 μM). *p<0.05 by one way ANOVA.

Figure 9. I-JIP (30,100 μM) and SP600125 (20 μM) increase radiation-induced VS cell apoptosis. *p<0.05 by one way ANOVA.

Key Research Accomplishments:

1- Demonstration that ErbB2 constitutively resides in lipid rafts in VS cells and translocates into lipid rafts in denervated Schwann cells, correlated with loss of merlin function.

2- Demonstration that protein kinase A phosphorylates merlin in Schwann cells *in vitro* and *in vivo* following denervation, correlated with proliferation and movement of ErbB2 into lipid rafts.

3- Demonstration that VS cells are relatively radioresistant. Inhibition of ErbB2 signaling further increases their radioresistance while activation of ErbB2 promotes radiosensitivity.

4- Demonstration of persistent activation of JNK in VS cells that promotes cell proliferation and survival by reducing oxidative stress. Further, persistent JNK activity accounts, at least in part, for the radioresistance of VS cells.

Reportable Outcomes:

Abstracts/Presentations:

Hansen, MR, Clark, JJ, Gantz, BJ, Goswami, PC. Effects of ErbB2 signaling on the response of vestibular schwannoma cells to γ-irradiation. Triologic Society, Orlando, Florida, May 2008—Fowler Award for Best Basic Science Thesis.

Woodson, EA, Clark, JJ, Xu, N, Provenzano, MJ, Hansen, MR. Constitutive ERK and PI3-K activity each promote proliferation in vestibular schwannoma cells while constitutive JNK activity and p75NTR signaling protect against apoptosis. Association for Research in Otolaryngology, Phoenix, Arizona, February 2008.

Clark, JJ, Brown, KD, Gantz, BJ, Hansen MR. Contribution of ErbB2 signaling to vestibular schwannoma cell proliferation and radiosensitivity. 5th International Conference on Vestibular Schwannomas and Other CPA Lesions, Barcelona, Spain, June 2007

Brown, KD, Clark, J, Hansen MR. Differential lipid raft localization of ErbB2 in vestibular schwannoma cells and Schwann cells. Combined Sections Meeting, Triologic Society, Marco Island, Florida, February 2007.

Brown, KD, Clark, J, Hansen MR. Differential lipid raft localization of ErbB2 in vestibular schwannoma cells and Schwann cells. Association for Research in Otolaryngology, Denver, Colorado, February 2007.

Manuscripts:

Hansen, MR, Clark, JJ, Gantz, BJ, Goswami, PC. (2008) Effects of ErbB2 signaling on the response of vestibular schwannoma cells to γ-irradiation. Laryngoscope, 118(6):1023-30.

Brown, KD, Hansen, MR. (2008) Lipid raft localization of erbB2 in vestibular schwannoma and Schwann cells. Otol Neurotol, 29(1):79-85.

Funding applied for based on work supported by this award:

Plastic Surgery Education Foundation/AAONHS   Richard Gurgel (PI)
Contribution of merlin inactivation by protein kinase A to facial nerve Schwann cell regenerative responses
The objectives are to determine the role of protein kinase A in the inactivation of merlin and Schwann cell mitosis following facial nerve injury.

Status: funded

NIH/NIDCD   Marlan Hansen (PI)
Contribution of c-Jun N-terminal kinase activity to vestibular schwannoma growth

The objectives are to determine the mechanisms leading to persistent c-Jun N-terminal kinase activity in vestibular schwannoma and the cellular consequences of inhibiting this activity.

Status: original application unfunded, resubmitted Nov. 4, 2008

Conclusions:

In summary, we have shown that VS cells, which lack functional merlin, constitutively express activated ErbB2 in lipid rafts contributing to their proliferative potential. Furthermore, protein kinase A inactivates merlin by phosphorylation in SCs *in vitro* and *in vivo* following denervation, correlated with movement of ErbB2 into lipids rafts and re-entry into the cell cycle. VS cells are relatively radioresistance, due at least in part, to persistent JNK activity which promotes cell survival and limits oxidative stress. Finally, inhibition of ErbB2 reduces VS cell radiosensitivity whereas activation of ErbB2 enhances radiosensitivity, likely by regulating cell proliferation.

References:

1. Brown KD, Hansen MR. Lipid raft localization of erbB2 in vestibular schwannoma and Schwann cells. *Otol Neurotol.* 2008;29(1):79-85.
2. Hansen MR, Clark JJ, Gantz BJ, Goswami PC. Effects of ErbB2 signaling on the response of vestibular schwannoma cells to gamma-irradiation. *Laryngoscope.* Jun 2008;118(6):1023-1030.
3. Clark JJ, Provenzano M, Diggelmann HR, Xu N, Hansen SS, Hansen MR. The ErbB inhibitors trastuzumab and erlotinib inhibit growth of vestibular schwannoma xenografts in nude mice: a preliminary study. *Otol Neurotol.* Sep 2008;29(6):846-853.

Lipid Raft Localization of ErbB2 in Vestibular Schwannoma and Schwann Cells

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Hypothesis: ErbB2 resides in lipid rafts (regions of receptor regulation) in vestibular schwannoma (VS) cells.
Background: ErbB2 is a growth factor receptor critical for Schwann cell (SC) proliferation and development. ErbB2 localization and activity may be regulated by merlin, an adaptor protein deficient in VS. Lipid rafts are microdomains in the plasma membrane that amplify and regulate receptor signaling. Persistence of erbB2 in lipid rafts in VS due to merlin deficiency may explain increased VS cell growth.
Methods: Protein extracts from VS or rat sciatic nerve (proximal or distal to a crush injury) were isolated into lipid raft and nonraft fractions and immunoblotted for erbB2, phosphorylated erbB2, and merlin (for sciatic nerve). Cultured VS cells were probed with anti-erbB2 antibody and a lipid raft marker, cholera toxin B (CTB).

Results: ErbB2 moves to lipid rafts in proliferating SCs and is persistently localized to lipid rafts in VS cells. ErbB2 is phosphorylated (activated) in lipid rafts. ErbB2 colocalized with CTB in cultured VS cells, confirming raft targeting. Merlin also persistently localized to lipid rafts in SCs, and its relative phosphorylation increased in proliferating cells.
Conclusion: Lipid raft localization of erbB2 in proliferating SCs and in VS cells supports a critical role for lipid rafts in amplifying/regulating erbB2 signaling. Merlin resides in lipid rafts in SCs, and its phosphorylation increases in proliferating SCs, suggesting it regulates cell proliferation within lipid rafts. The absence of merlin in VS may therefore lead to persistent erbB2 localization to lipid rafts and increased cell proliferation.
Key Words: Vestibular schwannoma—ErbB2—Merlin—Signal transduction—Lipid rafts.
Otol Neurotol **29:**79–85, 2008.

Vestibular schwannomas (VSs) represent benign neoplasms arising from Schwann cells (SCs) of the vestibular nerves. Two forms of the disease exist, a sporadic form and a form associated with the genetic disease neurofibromatosis type 2 (NF-2). Both forms share in common mutations in the *NF2* gene known as merlin or schwannomin (1). The merlin protein shares a high degree of homology with the ezrin-radixin-moesin family of proteins. This family of proteins functions as intermediates that integrate signaling between cell surface scaffolding proteins and cytoplasmic signaling cascades [reviewed in Bretscher et al. (2)]. Specifically, merlin appears to mediate contact inhibition of cell growth (3,4).

Of relevance to SC tumorigenesis, merlin has been implicated in regulating the subcellular localization and activity of the glial growth factor receptor, erbB2. In SCs, heterodimers composed of erbB2 and erbB3 function as receptors for the glial growth factor, neuregulin 1 (NRG1) (5). NRG1-induced dimerization of this pair leads to receptor phosphorylation followed by activation of intracellular pathways critical for SC survival and proliferation. These include the mitogen-activated protein kinase and phosphatidylinositol-3 kinase pathways (6). In the absence of erbB2 and erbB3, SCs fail to develop (7–9). ErbB2 signaling also contributes to the proliferation of SCs following axotomy, indicating that SCs critically depend on this receptor for normal development and proliferation (10).

In addition to its role in SC development and response to injury, erbB2 signaling contributes to the development and progression of SC neoplasms. Overexpression of NRG1 results in malignant peripheral nerve sheath tumors in transgenic mice, and human malignant peripheral nerve sheath tumor proliferation depends on erbB signaling (11,12). Of particular interest, recent investigations revealed that constitutive erbB2 activity contributes

to VS proliferation in vitro, further supporting a central role for erbB2 in human SC tumorigenesis (13–16).

Signaling by receptor tyrosine kinases, such as erbB2, is regulated at multiple levels including expression, degradation, access to ligand, and, in particular, subcellular localization. Cholesterol-enriched microdomains of the cell membrane, known as lipid rafts, concentrate receptors with intermediate molecules critical for amplifying and regulating signaling pathways [reviewed in Hancock (17)]. Cell surface receptors traffic in and out of these microdomains as they are engaged by their respective ligands, initiate their signaling cascades, and are ultimately removed. ErbB2 localizes to lipid rafts in other cell systems, suggesting that it may intermittently reside in lipid rafts in SCs and VS cells (18,19). Likewise, merlin localizes to lipid rafts in fibroblasts and has been implicated in regulating the intracellular localization and activity of receptor tyrosine kinases, including cell contact–dependent sequestration of erbB2 in SCs (4,20). These observations suggest a model wherein merlin regulates SC proliferation by regulating the subcellular localization of erbB2, among other molecules, in response to cell-cell contact cues (4).

Aberrant localization of growth-promoting receptors in lipid rafts may predispose to neoplasia (21). Persistent localization of erbB2 to lipid rafts (due to merlin deficiency) may therefore contribute to the dysregulated growth of VS cells. Here we evaluate the extent to which erbB2 localizes to lipid rafts in quiescent and proliferating SCs and in VS cells.

MATERIALS AND METHODS

Vestibular Schwannoma Collection

All patients provided written, informed consent for use of tumor harvested at the time of surgery. The institutional review board at the University of Iowa approved the study protocol. VS specimens were collected at the time of surgical removal and placed in ice-cold culture media (Dulbecco modified Eagle medium with 10% fetal calf serum and N2 additives) until either soluble and insoluble fractions were isolated (see below) or cells were cultured. A total of 8 tumors (sporadic form of disease) were examined (4 tumors for Western blots and 4 tumors for immunofluorescence studies).

Rat Sciatic Nerve Collection

The institutional animal care and use committee at the University of Iowa approved all protocols used in the study. Previous work has demonstrated increased erbB2 expression in denervated, proliferating rat sciatic nerve SCs beginning at 3 days after axotomy (10). We therefore selected 4 days as the period to evaluate erbB2 localization in proliferating SCs. Adult female Sprague-Dawley rats were anesthetized with ketamine/xylazine, and the sciatic nerve was exposed in the mid–gluteal region. The sciatic nerve was crushed for 10 seconds with a small hemostat (hematoma formation at the crush site facilitated easy future identification of this site), and the wound was closed. Four days later, the animals were killed, the operative site reopened, and proximal and distal nerve segments to the crush site collected. These segments were then pooled in 3 separate experiments of 3 animals each. Protein

extracts for Western blotting were isolated into soluble and insoluble fractions as described below.

Primary Vestibular Schwannoma Cell Culture

The culture of human VS cells has been previously described (14). Briefly VS specimens were cut into approximately 1-mm pieces and digested in collagenase and trypsin. They were then further dissociated by trituration through small-bore pipettes. The cells were initially resuspended in culture media (Dulbecco modified Eagle medium with 10% fetal calf serum and N2 additives) and plated onto 4-well tissue culture slides precoated with polyornithine and laminin. The cells were maintained in serum-free N2 media, following the initial media exchange until they reached greater than 70% confluency.

Bromodeoxyuridine Labeling of Sciatic Nerve

Four days following sciatic nerve crush injury, animals received 4 injections of bromodeoxyuridine (BrdU; 10 mg/mL in phosphate-buffered saline [PBS], 50 μg/g weight intraperitoneally) spaced over a 24-hour interval. The animals were killed, perfused with 4% paraformaldehyde transcardiac, and the distal and proximal sciatic nerve segments were harvested and fixed in 4% paraformaldehyde for 20 minutes. The nerve segments were stripped of the epineurium, and the fascicles were gently teased apart. Following treatment with 2 N HCl, the nerve segments were permeabilized with 0.8% Triton-X100 in PBS, blocked, and immunostained with anti-BrdU monoclonal antibody (1:1,000, clone G3G4, hybridoma core, University of Iowa) and rabbit anti-S100 (1:800; Sigma, St. Louis, MO, USA) followed by Alexa 488– and Alexa 546–conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA). Nuclei were labeled with Hoescht 3342 (10 μg/mL; Sigma). BrdU labeling was quantified by counting the percent of BrdU-positive SCs (S100-positive) nuclei in 3 randomly selected fields for each nerve segment from 3 separate animals. Differences in the mean percent of BrdU-positive cells were determined by the 2-tailed Student's t test using Excel software (Microsoft Corp., Redmond, WA, USA). Nerve segments were imaged using Leica SP5 confocal microscope using Leica software (Leica Microsystems Inc., Bannockburn, IL, USA).

Western Blotting of Vestibular Schwannoma and Rat Sciatic Nerve Extracts (Isolation of Soluble and Insoluble Fractions)

Isolation of soluble and insoluble fractions as well as the equivalence of the insoluble fraction with lipid rafts has been previously described (21). After washing the tissue pellet (either VS or sciatic nerve) with ice-cold PBS, the pellet was resuspended in ice-cold Triton-X100 (TX-100) buffer (1% TX-100, 150 mmol/L NaCl, 20 mmol/L Tris, pH 7.4 plus). Minicomplete protease inhibitors as well as phosphatase inhibitors were added, as per manufacturer's instructions, to all lysis buffers (Roche Diagnostics Corp., Indianapolis, IN, USA). The tissue was then sonicated to break connective tissue, and cells were then allowed to incubate 30 minutes on ice to allow dissolution of the nonlipid raft fraction. After this incubation, the lysates was centrifuged, and the supernatant removed. This supernatant constituted the soluble (nonlipid raft) fraction. The remaining pellet was then resuspended in an equal volume of sodium dodecyl sulfate (SDS) lysis buffer (0.5% SDS, 1% β-mercaptoethanol, 1% TX-100, 150 mmol/L NaCl, 20 mmol/L Tris, pH 7.4). By using equal volumes of buffer for each

isolated fraction, the relative amount of erbB2 in each fraction reflects its distribution between the soluble and insoluble fractions. Accurate comparisons as to the relative distribution (between soluble, nonlipid raft fractions and insoluble lipid raft fractions) of erbB2, phospho-erbB2, and merlin can then be made. The sample was then again sonicated to aid in resuspending the pellet from centrifugation and incubated for 30 minutes on ice to allow dissolution of lipid rafts. The resulting lysate constituted the insoluble (lipid raft) fraction. Both lysates then had an equivalent volume of SDS running buffer added. Lysates were then separated under denaturing conditions on a 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) gel and were subsequently transferred to nitrocellulose paper. Blots were then blocked with 5% dried milk in   Tris-buffered saline + Tween and immunoblotted with the following antibodies: anti-erbB2 (5 μg/mL AB-17; Lab Vision, Freemont, CA, USA), anti-phospho-erbB2 (2.5 μg/mL Ab-18, Lab Vision), anti-actin (1:5,000 Ab-5; BD Biosciences, Mississauga, ON, Canada), and anti–NF-2/schwannomin/merlin (1:10,000, sc-331; Santa Cruz Biotechnology, Santa Cruz, CA, USA). All blots were washed and then treated with either goat antimouse or goat antirabbit horseradish peroxidase–conjugated antibody at a 1:20,000 dilution (BioRad, Hercules, CA, USA). Blots were washed again and then developed with electrochemiluminescence solution, as per manufacturer's instructions (Pierce, Rockford, IL, USA) and exposed to film.

Immunostaining of Vestibular Schwannoma Cells

To verify colocalization of erbB2 with lipid rafts, VS cultures were washed twice with ice-cold PBS. Cells were then incubated with cholera toxin B (CTX-B)–Alexa Fluor 555 (red, 2.5 μg/mL) for 30 minutes at 4°C. Cholera toxin B binds GM1 ganglioside and is a marker for lipid rafts (Molecular Probes). Cells were light protected from this point on. After this incubation, cells were then washed 3 times with ice-cold PBS for 5 minutes each. Cells were then fixed with 4% paraformaldehyde/0.4% TX-100/PBS, pH 7.2, for 10 minutes. After fixation, cells were then washed 3 times with wash buffer (0.4% TX-100, PBS) for 5 minutes for each wash and blocked with blocking buffer (0.4% TX-100, 5% goat serum, 2% bovine serum albumin, PBS) for 30 minutes at room temperature. Cells were then washed once with wash buffer for 5 minutes. Mouse anti-erbB2 (Ab-2, 1:400; Oncogene Research Products, San Diego, CA, USA), diluted in blocking buffer, was then incubated overnight at 4°C. After 3 washes, Alexa Fluor 488 goat antimouse secondary antibody (1:1,000; Molecular Probes) was then incubated for 1 hour at room temperature. Cells were then washed 3 times and cover-slipped with 1 to 2 drops of Aquamount (Lerner Laboratories, Pittsburgh, PA, USA). Fluorescent digital images were captured using Zeiss LSM 510 confocal microscope. Images were prepared for publication with Adobe Photoshop (San Jose, CA, USA).

RESULTS

To better understand the role of erbB2 trafficking to lipid rafts in SC proliferation, we initially evaluated erbB2 localization in proliferating and quiescent normal SCs. Denervated SCs distal to the site of a sciatic nerve crush injury proliferate and increase expression of erbB2 within 3 days after injury, whereas those in the proximal segment remain quiescent (10). Therefore, we compared erbB2 localization to lipid rafts in nerve segments prox-

imal and distal to a crush injury. Four days after crushing the sciatic nerve, proximal and distal segments were collected and pooled from 3 animals in each of the 3 experiments. Protein extracts from the sciatic nerve segments were then isolated into TX-100 soluble and insoluble fractions. Equal volumes of lysate buffer were used to suspend these fractions to permit evaluation of the relative distribution of erbB2 between these fractions. These fractions were then separated by SDS-PAGE, transferred to nitrocellulose paper, and immunoblotted for erbB2, phosphorylated erbB2, and actin (to permit comparisons of protein content between proximal and distal segments). As shown in Figure 1C, erbB2 appeared exclusively in the nonlipid raft fraction (TX-100 soluble) in the proximal (nonproliferating) segment. The distal (proliferating) segment demonstrated an increase in overall erbB2 expression as previously reported (10) and also demonstrated the movement of erbB2 into the lipid raft (insoluble) fraction. This suggests that erbB2 moves to lipid rafts in proliferating SCs and supports the physiological importance of this localization in dividing cells. Furthermore, erbB2 is phosphorylated in the lipid raft fraction (Fig. 1D). As expected, the cytosolic protein actin remained in the TX-100 fraction verifying the adequacy of the TX-100 extraction (Fig. 1E). Proliferation of SCs in the distal segment was verified by BrdU labeling (Fig. 1, A and B). Together these data suggest that localization of erbB2 into lipid rafts is an important feature of SC proliferation and that erbB2 is activated in lipid rafts as demonstrated by its phosphorylation within these fractions.

Previous studies have suggested that merlin regulates erbB2 trafficking in SCs as merlin is present in erbB2 immunoprecipitates from SCs (4). Furthermore, merlin resides within lipid rafts in cultured fibroblasts (20). The data above demonstrate inducible erbB2 trafficking to lipid rafts in SCs that have lost axonal contact and re-enter the cell cycle. We then sought to determine if merlin also localizes to lipid rafts in SCs and if that localization varied between quiescent and proliferating nerve segments. TX-100 soluble and insoluble fractions derived from sciatic nerve segments proximal and distal to the crush injury were separated by SDS-PAGE, transferred to nitrocellulose paper, and immunoblotted for merlin. Merlin has been previously demonstrated to present as 2 dominant bands with the upper band representing its phosphorylated form (22,23). Merlin was exclusively found in the lipid raft (insoluble) fraction in rat sciatic nerve in both the distal (proliferating) and proximal (quiescent) segments of the nerve (Fig. 2). This finding was confirmed in 3 replicate experiments and is consistent with previous reports in other cell systems (20). Thus, merlin constitutively localizes to lipid rafts in normal SCs, implying that it likely plays a fundamental role in regulating the trafficking of key signaling molecules into and out of these microdomains of the cell membrane. Interestingly, the relative amount of phosphorylated merlin is higher in proliferating nerve, consistent with previous reports
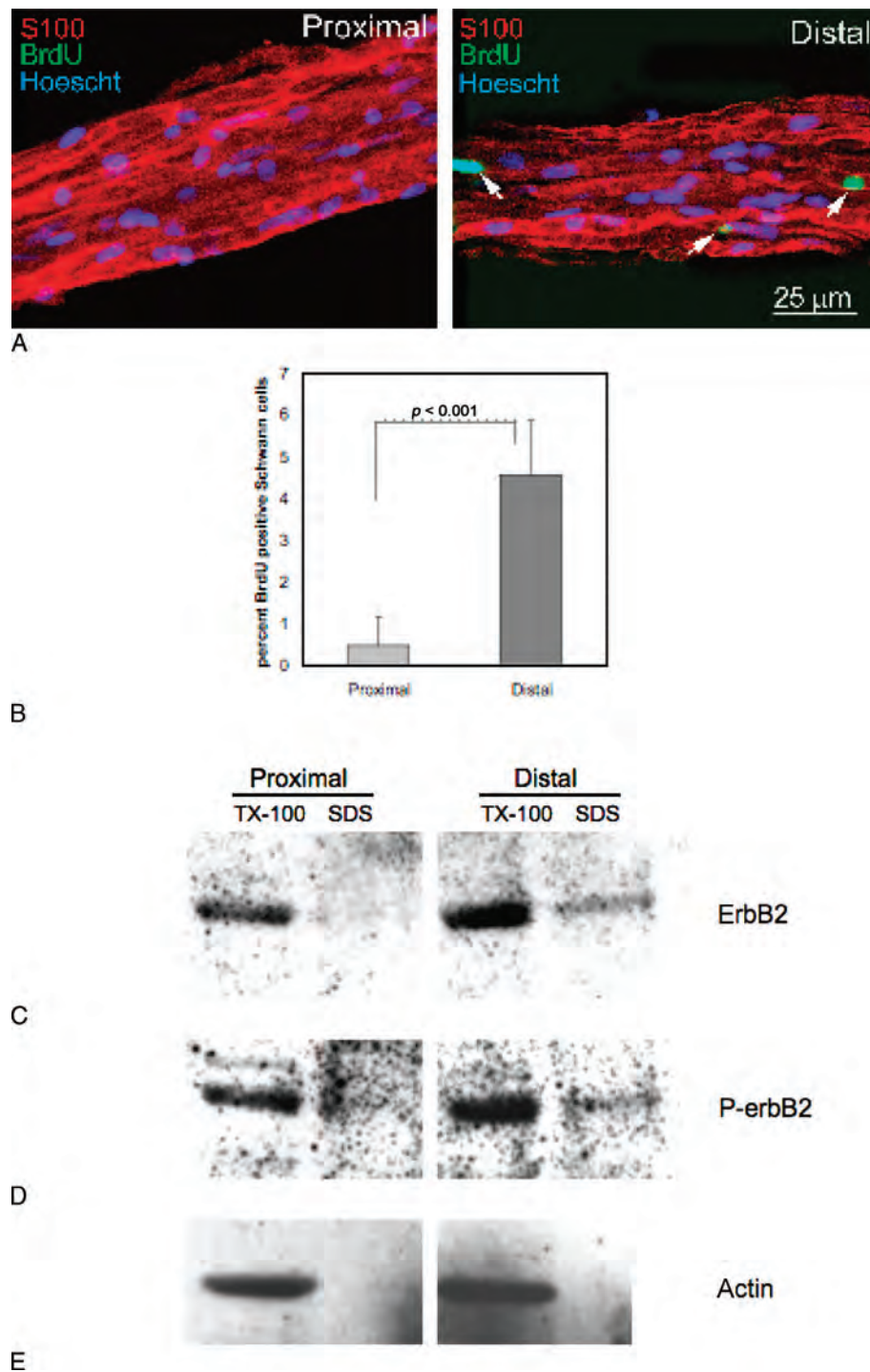
FIG. 1. Lipid raft localization of erbB2 in sciatic nerve. *A–B*, Four days after a crush injury, animals were labeled with BrdU as described in Materials and Methods. Proximal and distal segments were collected and immunostained with anti-BrdU monoclonal antibody (1:1,000, clone G3G4, hybridoma core, University of Iowa) and rabbit anti-S100 (1:800; Sigma) followed by Alexa 488- and Alexa 546-conjugated secondary antibodies (Molecular Probes). Nuclei were labeled with Hoescht 3342 (10 μg/mL; Sigma). BrdU labeling was quantified by counting the percent of BrdU-positive SC (S100 positive) nuclei in 3 randomly selected fields for each nerve segment from 3 separate animals. Differences in the mean percent of BrdU-positive cells were determined by the 2-tailed Student's *t* test and demonstrated significance at $p < 0.001$. *C–E*, Four days after a crush injury to the sciatic nerve, proximal (quiescent) and distal (proliferating) segments were collected, and soluble (TX-100/nonlipid raft) and insoluble (SDS/lipid raft) fractions were prepared. Fractions were separated by SDS-PAGE, transferred to nitrocellulose, and blotted for erbB2 (*C*) and phospho-erbB2 (*D*). Fractions were also blotted for actin (*E*) to verify equal protein loading and complete extraction of TX-100 soluble proteins. Data are representative of pooled nerve segments from 3 animals for each of 3 independent experiments.
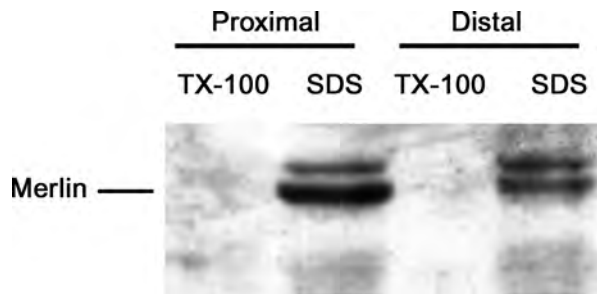
FIG. 2. Lipid raft localization of merlin in sciatic nerve. Four days after a crush injury to the sciatic nerve, proximal (quiescent) and distal (proliferating) segments were collected, and soluble (TX-100/nonlipid raft) and insoluble (SDS/lipid raft) fractions were prepared. Fractions were separated by SDS-PAGE, transferred to nitrocellulose, and blotted for merlin. The characteristic 2 dominant bands of merlin are seen. Data are representative of pooled nerve from 3 animals for each of 3 independent experiments.

indicating that phosphorylated merlin promotes cell proliferation (24).

Because merlin is implicated in erbB2 trafficking and is deficient in VS cells and because erbB2 activation contributes to VS proliferation, we next evaluated the lipid raft distribution of erbB2 in acutely resected human VS. VS tissue was harvested and isolated into TX-100 soluble and insoluble fractions (21,25). As demonstrated previously, the insoluble fraction corresponds to the lipid raft–containing fraction of the cell (21). Each of these fractions was then separated by SDS-PAGE, transferred to nitrocellulose paper, and immunoblotted for erbB2. The blot was then stripped and reprobed with an anti–phosphorylated erbB2 antibody. Equal volumes of buffer were used for each isolated fraction, and equal volumes were loaded into the gel for each fraction; therefore, the relative amount of erbB2 in each fraction reflects its distribution between the soluble and insoluble fractions. Remarkably, erbB2 exclusively localized within the lipid raft (TX-100 insoluble) fraction in VS tissue (Fig. 3A). The phosphorylated form of erbB2 is likewise localized to the lipid raft fraction (Fig. 3B), suggesting that the erbB2 in TX-100 insoluble fraction exists in an activated state consistent with the observation of constitutive erbB2 activation in VS tissue (14). Similar findings were seen in VS specimens from 4 patients. These data together demonstrate that in VS, which lacks functional merlin, activated erbB2 localizes to lipid rafts to a greater extent than that seen in denervated SCs and suggests the possibility that constitutive erbB2 localization in lipid rafts in VS contributes to their growth potential.

Although the TX-100 insoluble fraction has been previously demonstrated to contain lipid rafts, other subcellular domains such as caveolae and insoluble cytoskeletal components are likewise present within this fraction. To verify that erbB2 in the TX-100 insoluble fraction is due to its distribution into lipid rafts, we used colocalization studies with fluorophore-labeled CTX-B, which specifically binds to GM1 ganglioside

and is a reliable marker for lipid rafts and anti-erbB2 immunofluorescence. VS cultures near confluency were labeled with Alexa Fluor 555–conjugated CTB (red) followed by fixation and immunolabeling with anti-erbB2 and an Alexa Fluor 488 secondary antibody (green). Cells were imaged with confocal microscopy, and images were combined to evaluate for overlap of the fluorophores. As shown in Figure 4, erbB2 immunofluorescence is predominantly restricted to the cell membrane where it extensively overlaps with CTX-B labeling, confirming that erbB2 localization to the TX-100 insoluble (lipid raft) fraction is due to its localization to lipid rafts.

DISCUSSION

Lipid rafts are proposed to serve as hubs of activity by which signals of cell growth are amplified and/or regulated before being transduced into the cell (26). Merlin is the deficient or mutated protein in VS, and previous studies suggest that merlin may localize to lipid rafts in fibroblasts (1,20). Similarly, in other cell systems, erbB2 appears to localize to lipid rafts (18). ErbB2 and its binding partner erbB3 have been proposed to promote the proliferation of schwannoma cells (13–16). We therefore hypothesized that differences in lipid raft localization of erbB2 may, in part, explain the constitutive activation of erbB2 in VS.

Our evaluation revealed that erbB2 is constitutively localized to lipid rafts in VS tissue. This was demonstrated both biochemically by extracting a lipid raft fraction (Fig. 3) as well as by imaging, demonstrating that erbB2 colocalizes with CTX-B, a marker for lipid rafts
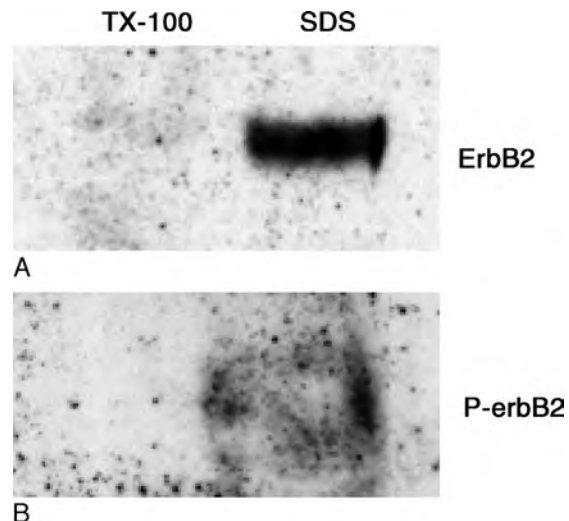

FIG. 3. Lipid raft localization of erbB2 in VS cells. Soluble (TX-100/nonlipid raft) and insoluble (SDS/lipid raft) fractions were prepared from freshly harvested tumor specimens. Fractions were separated by SDS-PAGE, transferred to nitrocellulose, and blotted for erbB2 (*A*) and phospho-erbB2 (*B*). Data are representative of 4 independent experiments performed on 4 separate tumors.
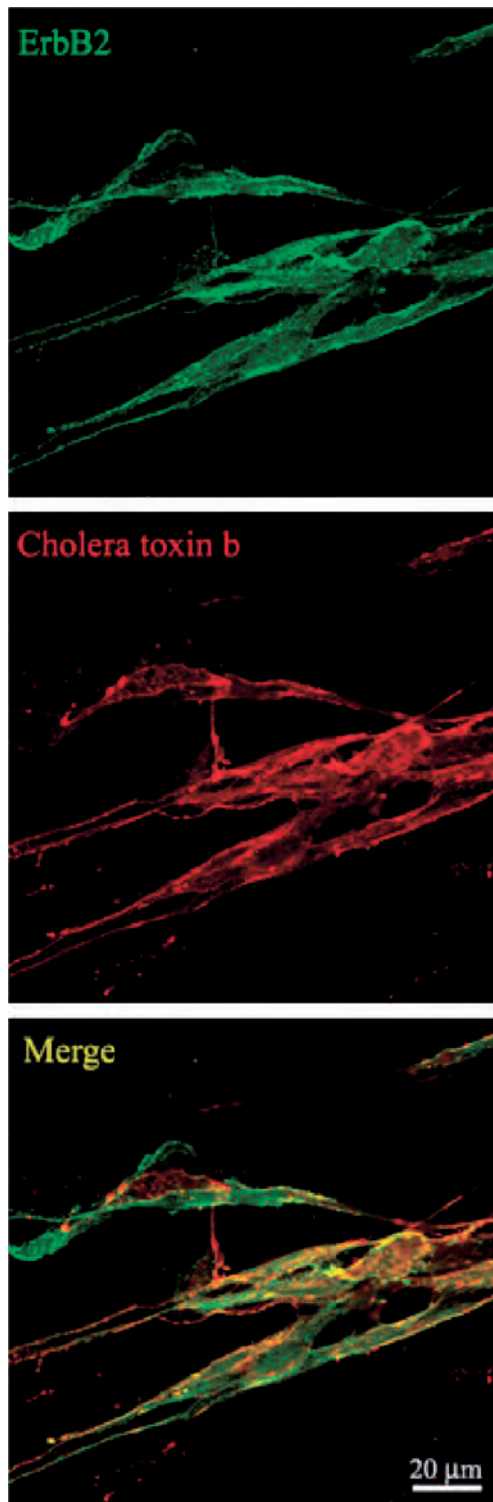
FIG. 4. Colocalization of erbB2 with lipid rafts. VS cells were cultured and labeled with Alexa Fluor 555–conjugated CTX-B (*red*), a lipid raft marker, and anti-erbB2 with Alexa Fluor 488 secondary antibody (*green*). Fluorescence was detected with confocal microscopy. Overlap of the *red* and *green* labeling produces a *yellow* color indicating colocalization of the molecules. Data are representative of 4 independent experiments performed on 4 separate tumors.

(Fig. 4). Activation of erbB2 in this fraction is demonstrated by its phosphorylation (Fig. 3). ErbB2 has previously been suggested to play a key role in SC proliferation as well as proliferation of VS cells (10,13,14,27). The constitutive presence of activated erbB2 in lipid rafts (which are an axis for growth-promoting receptors to initiate their signal transduction cascades) is a novel finding and provides a key piece of evidence for how disordered growth may occur in VS cells.

The significance of this finding is further underscored by our demonstration that SCs in proliferating sciatic nerve distal to a crush injury site not only increase erbB2 expression, but also translocate erbB2 to the insoluble lipid raft fraction (Fig. 1). This correlation between proliferating SCs and the presence of erbB2 in lipid rafts suggests that movement of erbB2 into lipid rafts following loss of axonal contact is a physiological response of SCs that promotes proliferation. Taken with our observation of constitutive erbB2 localization to lipid rafts in VS cells, these results suggest a mechanism for constitutive erbB2 signaling in VS cells that contributes to their proliferative potential (14).

Merlin exists in 2 states: an open, phosphorylated form that promotes cell proliferation and growth and a closed hypophosphorylated form that inhibits cell growth (24). The presence of merlin in lipid rafts has been previously suggested to be crucial for the ability of merlin to intercede and disrupt positive growth cues emanating from the plasma membrane (20). We demonstrate in our work for the first time that merlin is localized to lipid rafts in SCs and that its relative phosphorylation increases within this fraction in proliferating SCs. These data together suggest that merlin regulation of receptor kinases such as ErbB2 occurs in lipid rafts. Merlin interacts with the erbB2 binding protein, erbin, via the anchoring protein EBP50 (28), and has been proposed to regulate the membrane localization of erbB2 in SCs in response to cell-cell contact signals (4). In this model, merlin mediates the assembly of transmembrane and intracellular signaling hubs based on cell-cell contact signals (4). In subconfluent SCs in vitro or denervated SCs in vivo, merlin is phosphorylated and "open." In this state, merlin interacts with several transmembrane, scaffolding and signaling molecules including erbB2, facilitating the assembly of a complex of signaling molecules. In confluent cells in vitro or in the presence of axons, merlin is hypophosphorylated and closed. This leads to disruption of the signaling complex, sequestration of erbB2 from lipid rafts and cell cycle arrest. As VS cells lack functional merlin (1), they would be unable to accomplish this disruption of signaling complexes leading to a state of constitutive activation in which erbB2 remains localized in lipid rafts despite increased cell density. Although further study is necessary to fully validate this model, it offers an appealing explanation for how erbB2 activity could contribute to the disordered growth of VS cells.

Together, these data demonstrate that activated erbB2 is constitutively localized to lipid rafts in VS in both whole tissue and cell culture. ErbB2 is also inducibly localized to rafts in denervated, proliferating SC sciatic nerve, suggesting a critical role for erbB2 in SC proliferation. Merlin may regulate erbB2 trafficking in lipid rafts and its absence in VS cells may, in part, explain the increased growth potential of VS cells.

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CANDIDATE'S THESIS

Fowler Award Presentation

Effects of ErbB2 Signaling on the Response of Vestibular Schwannoma Cells to γ-Irradiation

Marlan R. Hansen, MD; J. Jason Clark, MS; Bruce J. Gantz, MD; Prabhat C. Goswami, PhD

Objective: For vestibular schwannomas (VSs) that require treatment, options are limited to microsurgery or irradiation (IR). Development of alternative therapies that augment or replace microsurgery or IR would benefit patients not suitable for current therapies. This study explored the ability of ErbB2 inhibitors to modulate the effects of IR on VS cells.

Study Design: Prospective study using primary cultures derived from human VSs.

Methods: Primary cultures of VS cells were derived from acutely resected tumors. Cultures received single escalating doses (15–40 Gy) of γ-irradiation from a ^{137}Cs γ-irradiation source. Cell proliferation was determined by BrdU uptake and apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Trastuzumab (Herceptin) and PD158780 were independently used to inhibit ErbB2 signaling while neuregulin-1β (NRG-1) was used to activate ErbB2.

Results: IR induces VS cell cycle arrest and apoptosis in doses greater than 20 Gy, demonstrating that VS cells are relatively radioresistant. This radioresistance likely arises from their low proliferative capacity as a sublethal dose of IR (10 Gy) strongly induces deoxyribonucleic acid (DNA) damage evidenced by histone H2AX phosphorylation. Inhibition of ErbB2, which decreases VS cell proliferation, protects VS cells from radiation-induced apoptosis, while NRG-1, an ErbB2 ligand and VS cell mitogen, increases radiation-induced VS cell apoptosis.

Conclusions: Compared with many neoplastic conditions, VS cells are relatively radioresistant. The radio-protective effect of ErbB2 inhibitors implies that the sensitivity of VS cells to IR depends on their proliferative capacity. These results hold important implications for current and future treatment strategies.

Key Words: Proliferation, apoptosis, acoustic neuroma, radiotherapy, histone H2AX.

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INTRODUCTION

Vestibular schwannomas (VSs) represent benign neoplasms arising from the Schwann cells (SCs) within the vestibular nerves. Most occur as sporadic, isolated tumors; however, patients with neurofibromatosis type 2 (NF2) develop multiple intracranial and spinal neoplasms, including bilateral VSs.[1] Management of VSs remains controversial. For many patients, observation with serial imaging to monitor for further growth suffices.[2,3] For patients who elect or require treatment, options are limited to microsurgical resection or irradiation (IR).[4] Estimates predict that in the coming decade, most VSs will be managed with IR.[5] Typically, IR is provided as stereotactic radiosurgery (SRS) in a single dose delivered from a gamma knife or linear accelerator (LINAC) or as fractionated stereotactic radiotherapy (FSR) delivered in fractionated doses.[6] Both microsurgery and SRS/FSR are generally well tolerated, yet occasionally result in significant morbidity and, in rare cases, even malignant transformation.[7–11] Further, some patients are not good candidates for either microsurgery or SRS/FSR. Understanding the mechanisms that regulate VS growth and their response to IR will hopefully lead to the development of effective alternative therapies that specifically limit schwannoma growth or increase their response to current therapies.

***Vestibular schwannomas result from defects in the tumor suppressor gene,* merlin.** Both sporadic and familial (NF2) forms of VSs are associated with defects in the tumor suppressor gene, *schwannomin/merlin*.[12–14] The merlin protein shares a high degree of homology to the ezrin-radixin-moesin family of proteins believed to function by associating transmembrane and signaling molecules with

cytoskeletal actin, and so affecting cell-cell attachments, cell motility, and localization of cell signaling molecules.[15] Recent investigations have begun to identify mechanisms by which lack of merlin function may promote tumor growth. Merlin inhibits several intracellular signals implicated in cell proliferation and tumor formation, including Ras, Rac1/Cdc42, Raf, p21-activated kinases 1 and 2, extracellular regulated kinase/mitogen activated protein kinase (ERK/MAPK), and phosphatidyl-inositol 3-kinase (PI3-K)/Akt.[15] In addition to its effects on intracellular signals, merlin also regulates receptor tyrosine kinase trafficking and activity, including platelet-derived growth factor receptor[16] and ErbB2.[17,18] The extent to which these mechanisms contribute to SC neoplasia is unknown.

ErbB2 signaling contributes to Schwann cell development, proliferation, survival, and tumorigenesis. ErbB2 and ErbB3 are members of the epidermal growth factor (EGF) family of receptor tyrosine kinases, and both are required for normal SC development and survival.[19,20] They function as heterodimeric receptors for neuregulin-1 (NRG1), a potent axonally-derived SC mitogen that is essential for normal SC development and survival.[21] NRG1-induced ErbB2 and ErbB3 phosphorylation leads to activation of intracellular signals, including PI3 K/Akt and ERKs, which are necessary for SC proliferation and survival.[21–25]

Similar to denervated SCs, VS cells constitutively express NRG1 and its receptors ErbB2 and ErbB3. In VS cells, ErbB2 resides in detergent-resistant microdomains of the cell membrane associated with enhanced signal transduction known as lipid rafts, are constitutively active, and promotes VS cell proliferation.[26–28] Additional lines of evidence suggest that constitutive NRG1:ErbB2 signaling contributes to SC neoplasia. Mice genetically engineered to overexpress NRG1β in SCs develop malignant SC tumors[29] and constitutive NRG1:ErbB signaling contributes to cell proliferation in malignant SC tumors.[30,31] Thus, NRG1:ErbB2 signaling offers a potential therapeutic target for VS intervention.[27]

Effects of irradiation on vestibular schwannomas. In an effort to avoid surgical complications, SRS/FSR are increasingly used in the management of VSs. SRS/FSR typically do not result in complete VS regression; rather, in most cases, they result in partial tumor reduction or prevent further growth.[32,33] With the recent rise in the number of VSs treated with SRS/FSR and with longer periods of follow-up, an increasing number of treatment failures are being reported[34] and tumors from NF2 patients may be particularly radioresistant.[35–37] The lack of complete tumor regression in most VSs and the continued growth of selected VSs following SRS/FSR highlight the fact that compared with many neoplastic conditions, VSs are relatively resistant to IR. Despite the dramatic rise in the number of VSs treated with SRS/FSR recently,[5] the effects of IR on VS cells themselves remain largely unknown. Further, there are no known reagents that modify the response of VS cells to IR.

Constitutive ErbB2 activity in VS cells raises the possibility of using ErbB2 inhibitors in the management of VSs, especially those in patients with NF2, which are less amenable to microsurgical resection or SRS/FSR.[35–37] In addition to VSs, ErbB2 contributes to the growth of several other neoplastic conditions, most notably, breast carcinoma where up to 30% overexpress ErbB2.[38] In these cells, ErbB2 signaling appears to confer a radioprotective effect, and concurrent treatment with ErbB2 inhibitors increases the radiosensitivity of the tumor cells.[39,40] VSs that grow after SRS demonstrate persistent ErbB2 activation,[26] suggesting that ErbB2 activity may contribute to their radioresistance. Alternatively, since dividing cells are most sensitive to IR, ErbB2 signaling could increase VS cell radiosensitivity by promoting proliferation.

This study sought to determine the extent to which ErbB2 signaling modulates the response of cultured VS cells to IR. The data show that inhibition of ErbB2, which decreases VS cell proliferation, reduces radiation-induced VS cell apoptosis. By contrast, the ErbB2 ligand, NRG-1, a VS cell mitogen, enhances VS cell radiosensitivity. These results demonstrate that the response of VS cells to IR depends on their proliferation rate, which is regulated by ErbB2 signaling, and have important implications for current and future VS management strategies.

METHODS

Vestibular Schwannoma Cultures

All patients provided written consent and the procedures for obtaining VS samples were approved by the Institutional Review Board. Primary VS cultures were prepared as has been previously described.[27] Briefly, acutely resected tumors were minced into ~1 mm³ fragments, treated with 0.25% trypsin and 0.1% collagenase for 30 to 40 minutes at 37°C, and dissociated by tituration through narrow bore-glass pipets. Cell suspensions were plated on four-well plastic culture slides (Nalge Nunc International, Rochester, NY) coated with poly-ornithine followed by laminin (20 μg/mL) in Dulbecco's modified Eagle's medium (DMEM) with N2 supplements (Sigma, St. Louis, MO), bovine insulin (Sigma, 10 μg/mL) and 10% fetal calf serum (FCS). The medium was exchanged 1 to 2 days later and the cells were subsequently maintained in serum-free conditions until used for experiments, typically after 7 to 10 days. Cultures were maintained in a humidified incubator with 6.0% CO_2 at 37°C. Trastuzumab (Herceptin, HCN 10 μg/mL), PD158780 (Calbiochem, San Diego, CA, 20 μmol/L), or neuregulin 1β (LabVision, Fremont, CA, 3 nM) was added to the indicated cultures 24 hours prior to irradiation and maintained throughout the duration of the experiment. A total of 10 VS cultures, each derived from separate patients, were used in these studies.

Immunocytochemistry

Following fixation with 4% paraformaldehyde, the cultures were washed in phosphate buffered saline (PBS) and permeabilized with 0.8% Triton-×100 in PBS for 15 minutes. Nonspecific antibody binding was blocked with 5% goat serum, 2% bovine serum albumin (BSA), in phosphate buffered saline (PBS) with 0.8% Triton-×100. The cultures were then treated with primary antibodies overnight at 4C and then rinsed three times in PBS with 0.8% Triton-×100. The following primary antibodies were used in various combinations: Rabbit polyclonal antiS100 antibody (Sigma, 1:800), monoclonal antiBrdU antibody (University of Iowa Hybridoma Bank, Iowa City, IA, clone G3G4, 1:1,000), and monoclonal antiphosphorylated Ser139 histone H2AX (Upstate Cell Signaling Solutions, Charlottesville, VA, 1:500). Secondary detection of primary antibody labeling was accomplished

using goat, antirabbit, and antimouse secondary antibodies conjugated to Alexa 488 or Alexa 568 (Invitrogen, Carlsbad, CA, 1:1,000). Following immunostaining, nuclei were stained with Hoescht 3342 (Sigma, 10 μg/mL) in PBS for 10 minutes at room temperature. Immunostaining was detected using an inverted Leica DMRIII microscope (Leica Microsystems, Bannockburn, IL) equipped with epifluorescence filters, and digital images were captured with a charge-coupled device Leica DFC 350FX camera (Leica Microsystems) using Leica FW4000 software. Images were analyzed in Image J (NIH, Bethesda. MD) and prepared for publication using Adobe PhotoShop (Adobe, San Jose, CA).

Determination of Vestibular Schwannoma Cell Proliferation

VS cultures were labeled with BrdU (Sigma, St. Louis, MO, 10 μg/mL) for 48 hours prior to fixation. Fixed cultures were treated with 2N HCl for 15 minutes prior to immunostaining and BrdU uptake was detected by immunostaining as above. The percent of BrdU positive VS cells (S100 positive) nuclei was determined by counting 10 randomly selected fields for each condition. Only S100 positive cells were scored. The average number of cells per field was 103.04 ± 4.23 (standard deviation). Since there is variability in the proliferation rate of individual tumors, the percent BrdU uptake was expressed as a percent of the control condition defined as 100%. The average percent of BrdU positive VS cells in the control condition was 13.17% ± 3.22 standard error of the mean (SEM). Each condition was repeated on at least three VS cultures derived from separate patients.

Determination of Vestibular Schwannoma Cell Apoptosis

Following fixation and immunostaining with antiS100, apoptotic cells were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using the In Situ Cell Death Detection Kit, TMR red kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Nuclei were labeled with Hoescht 3342 as above. The percent of apoptotic VS cell (S100 positive) nuclei was determined by counting 10 randomly selected fields for each condition. Criteria for scoring were a TUNEL-positive nucleus with typical condensed morphology in an S100 positive cell. The percent of apoptotic VS cells was expressed as a percent of the control condition defined as 100%. The average percent of apoptotic VS cells in the control condition was 4.65% ± 0.80 (SEM). Each condition was repeated on 3 or more VS cultures derived from separate patients.

Irradiation of Vestibular Schwannoma Cultures

Primary VS cultures were irradiated by using a cesium-137 gamma radiation source set at dose rate of 0.84 Gy/minute. Control cultures, receiving sham IR, were treated in an identical manner but were not exposed to radiation.

Statistical Analyses

Evaluation for statistical differences in mean percent apoptotic and percent BrdU-positive VS cells among the various conditions was performed by analysis of variance (ANOVA) with post hoc Hidak-Solm analysis using SigmaStat software (Systat Software, Richmond, CA). For evaluation of differences of the mean percent apoptotic VS cells in 30 Gy versus 30 Gy positiveNRG1, the Student t test was used.

RESULTS

Single Doses of γ-Irradiation of 30 Gy or Greater Result in VS Cell Apoptosis

The objective of this study was to determine the effects of ErbB2 signaling on the response of VS cells to IR.

Despite the widespread use of SRS/FSR to treat VSs, the effects of IR on VS cells are not well characterized. Thus, investigation of the effects of ErbB2 signaling on VS radiosensitivity first required definition of the response of VS cells to IR. Primary VS cultures derived from acutely resected tumors were used for these purposes. These cultures contain over 98% schwannoma cells as determined by S100 immunoreactivity (Figs. 1, 2, and 3). Since the dose response of VS cells to IR has not been well defined, we evaluated the response of cultures derived from eight separate VSs treated with escalating doses (15–40 Gy) of γ-irradiation from a ^{137}Cs γ-irradiation source. Control cultures received sham IR. The clonogenic assay remains the gold standard assay for radiation-induced cell death.[41]

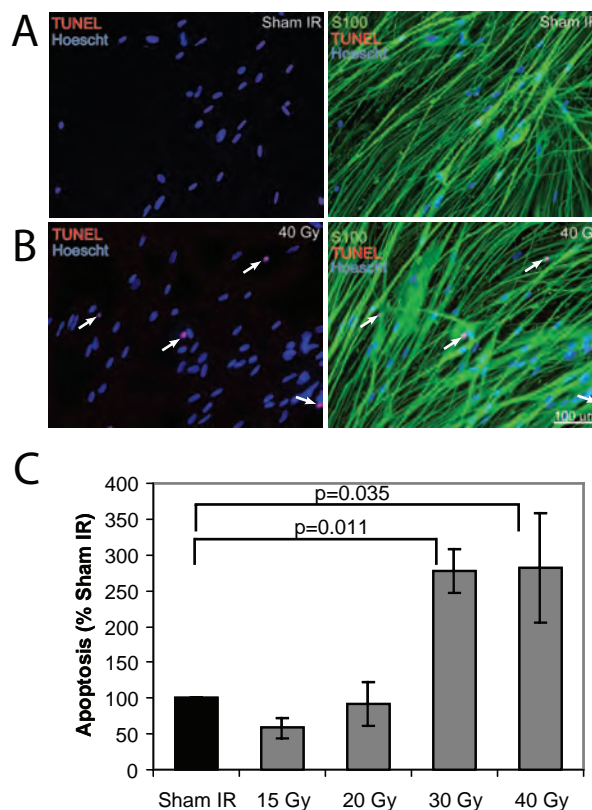Fig. 1. Vestibular schwannoma (VS) cell apoptosis is induced by γ-irradiation (IR). Primary VS cultures were irradiated with 15–40 Gy. Control cultures received sham IR. Seven days later, the cultures were fixed, immunolabeled with antiS100 antibodies followed by Alexa 488 secondary antibody (green) and labeled with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using TRITC-labeled dUTP (red). Nuclei were identified with Hoescht 3342 (blue). (A and B) Representative images from cultures receiving sham IR (A) or 40 Gy IR (B). Left panels: composite images of TUNEL (red) and Hoescht (blue) labeling. Right panels: composite images of antiSl00 (green), TUNEL, and Hoescht labeling. Arrows indicate TUNEL positive nuclei determined by overlap of red and blue channels. Scale bar = 100 μm. (C) Quantification of percent TUNEL-positive VS cells. The percent of TUNEL-positive VS cells in each condition was determined from 10 randomly selected fields and is expressed as a percent relative to cultures receiving sham IR, defined as 100%. Each condition was performed on at least three tumors derived from separate patients. Error bars present standard error of the mean (SEM). P values indicate significant differences by analysis of variance (ANOVA) with post hoc Hidak-Solm analysis.
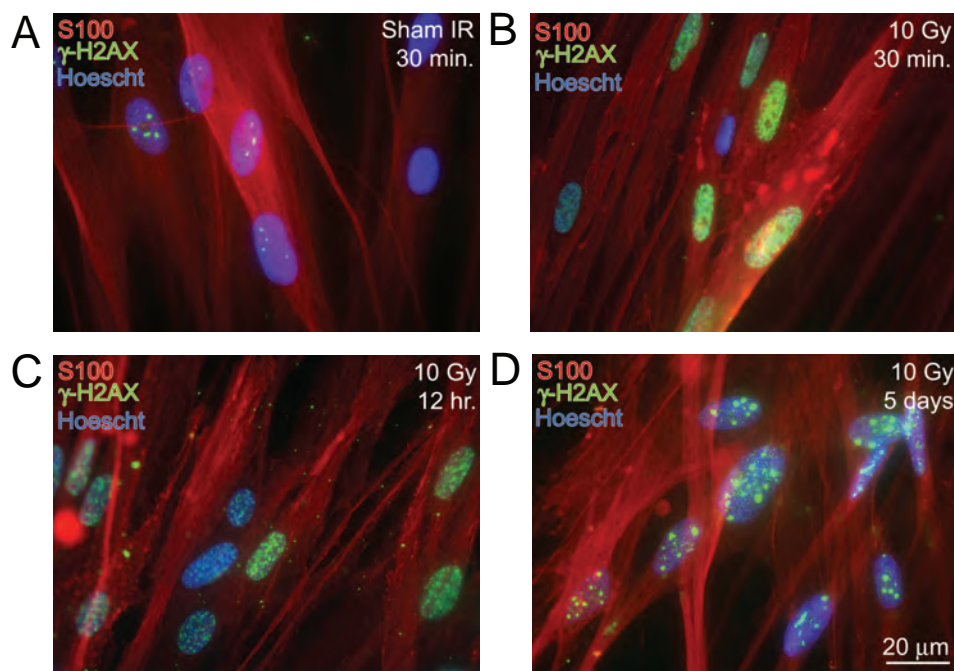
Fig. 2. Ten Gy γ-irradiation (IR) induces histone 2A phosphorylation (γ-H2AX) in vestibular schwannoma (VS) cells. Primary VS cultures received sham IR (A) or 10 Gy IR and were fixed 30 minutes (B), 12 hours (C), or 5 days (D) later. The cultures were immunolabeled with a monoclonal antibody that recognizes γ-H2AX followed by an Alexa 488 secondary antibody (green) and antiS100 antibody followed by Alexa 568 secondary antibody (red). Nuclei were identified with Hoescht staining. Scale bar = 20 μm.

However, VS cells are not transformed and do not form clones, making this assay impossible. Therefore, we used apoptosis as a marker for radiation-induced cell death.[41] Irradiation also induces necrotic cell death; however, since ErbB2 signaling regulates apoptosis in Schwann cells,[42] we focused on defining the apoptotic response of VS cells to IR.

Seven days following IR, the cultures were fixed and immunostained with antiS100 antibodies followed by an Alexa 488 (green) conjugated secondary antibody to specifically identify VS cells. The cells were then labeled with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). TUNEL identifies apoptotic cells in situ by using terminal deoxynucleotidyl transferase (TdT) to transfer TRITC labeled dUTP (red) to strand breaks of cleaved deoxyribonucleic acid (DNA) (Fig. 1). Nuclei were identified with Hoescht 3342 (blue) labeling. The percent TUNEL positive VS cell (S100 positive) nuclei was determined from 10 randomly selected fields for each well. All TUNEL-positive nuclei were condensed, typical of apoptotic cell death. Thirty Gy and 40 Gy resulted in a nearly threefold increase in VS cell apoptosis compared with cultures receiving sham IR ($277\% \pm 30$ and $282\% \pm 77$, respectively, mean $\pm$ SEM), which was statistically significant ($P < .05$), while the percent of apoptotic VS cells in cultures treated with 20 Gy or less was not significantly different from those in cultures receiving sham IR (Fig. 1). These results suggest that, compared with most malignant cells, VS cells are relatively resistant to IR.

Two possible explanations for the relative radioresistance of VS cells are that either higher doses of IR are needed to damage the VS cells' DNA or that the cells are capable of repairing DNA damage prior to re-entering cell cycle.[41] In the latter case, cells that successfully repair the damaged DNA prior to re-entering cell cycle would be less likely to undergo apoptosis. To determine if sublethal doses of IR are capable of damaging VS cell DNA, we immunostained cultures treated with 10 Gy IR with an antibody that recognizes phosphorylated histone H2AX (γ-H2AX). H2AX is phosphorylated following double-stranded breaks in DNA and is a sensitive indicator of radiation-induced DNA damage.[43] Within 30 minutes of IR, over 90% VS cells exhibit robust γ-H2AX immunoreactivity compared with cells receiving sham IR (Fig. 2). The intensity of the γ-H2AX immunoreactivity declines and becomes more punctate, but persists over the subsequent 5 days in over 80% of the VS cells (Fig. 2). These results demonstrate that sublethal doses of IR damage VS cell DNA and suggest that VS cells are capable of repairing damaged DNA due to sublethal doses of IR prior to re-entering cell cycle.

40 Gy γ-Irradiation Reduces Vestibular Schwannoma Cell Proliferation

A main consequence of SRS/FSR on VSs in vivo appears to be a lack of further tumor growth. One possible explanation for this decreased growth rate is that IR causes cell cycle arrest. We therefore asked to what extent IR induced cell cycle arrest in cultured VS cells. Primary VS cultures were irradiated with escalating doses of IR. Five days later, the cultures were treated with BrdU (10 μmol/L) for a further 48 hours. The cultures were then fixed and immunostained with antiBrdU and antiS100 antibodies. Nuclei were stained with Hoescht (Sigma-Aldrich, St. Louis, MO). The percent of BrdU positive VS cell (S100 positive) nuclei was determined from 10 randomly selected fields for each well. A total of seven VS tumors were used in these studies.

As shown in Figure 3, 40 Gy IR significantly reduced the percent of BrdU positive VS cells to $68\% \pm 15\%$ (mean $\pm$ SEM) of cultures receiving sham IR ($P = .035$),
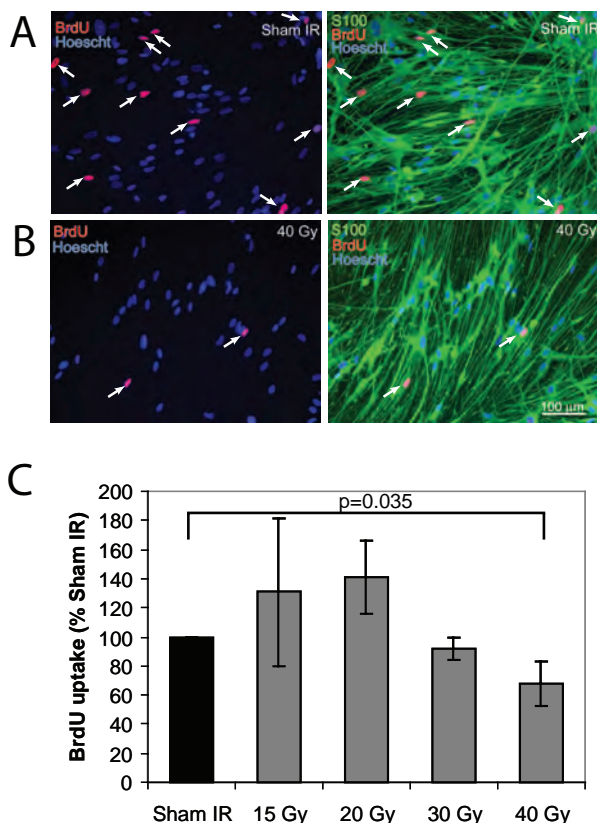
Fig. 3. Forty Gy γ-irradiation (IR) reduces vestibular schwannoma (VS) cell proliferation. Primary VS cultures were irradiated with doses from 10 to 40 Gy. Control cultures received sham IR. Five days following irradiation the cultures were treated with BrdU (10 μmol/L) for an additional 48 hours, fixed, and immunolabeled with antiS100 antibodies followed by Alexa 488 secondary antibody (green) and antiBrdU monoclonal antibody followed by an Alexa 568 secondary antibody (red). Nuclei were identified with Hoescht 3342 (blue). (A and B) Representative images from cultures receiving sham IR (A) or 40 Gy IR (B). Left panels: composite images of BrdU (red) and Hoescht (blue) labeling. Right panels: composite images of antiS100 (green), BrdU, and Hoescht labeling. Arrows indicate BrdU positive nuclei determined by overlap of red and blue channels. Scale bar = 100 μm. (C) Quantification of percent BrdU positive VS cells. The percent BrdU positive VS cells in each condition was determined from 10 randomly selected fields and is expressed as a percent relative to cultures receiving sham IR, defined as 100%. Each condition was performed on a minimum of five tumors derived from separate patients. Error bars present standard error of the mean (SEM). *P* value indicates significant difference by analysis of variance (ANOVA) with post hoc Hidak-Solm analysis.

while lower doses did not significantly reduce VS cell proliferation. Thus, 40 Gy IR reduces VS cell proliferation in vitro.

ErbB2 Inhibitors Reduce Vestibular Schwannoma Cell Proliferation and Protect Vestibular Schwannoma Cells From Irradiation-Induced Apoptosis

Compared with many malignancies, VSs are relatively resistant to IR.[44,45] One explanation for the relative radioresistance of VS cells is their low proliferation rate. Another possible contributing factor is constitutive activation of protective signaling pathways such as

those recruited by ErbB2.[26,27,40,46] Conversely, by increasing proliferation, ErbB2 activity could enhance the response of VS cells to IR. Therefore, we asked to what extent ErbB2 signaling modulated the response of VS cells to IR.

To inhibit ErbB2 signaling we used two separate molecules: 1) trastuzumab (Herceptin, HCN, a humanized antiErbB2 monoclonal antibody used to treat breast carcinomas that overexpress ErbB2, and 2) PD158780, a small molecule ErbB2 inhibitor. Both molecules inhibit ErbB2 signaling in cultured VS cells.[27] To determine if ErbB2 activity modulated VS radiosensitivity, VS cultures were treated with trastuzumab (100 μg/mL) or PD158780 (20 μmol/L) 24 hours prior to receiving 30 or 40 Gy of IR. We have previously shown that these doses effectively reduce VS cell proliferation and inhibit SC proliferation in response to neuregulin-1 (NRG-1), an ErbB2 ligand and SC mitogen. For cultures used in proliferation assays, BrdU was added for the final 48 hours in culture. Seven days following IR, the cultures were fixed and the percent of apoptotic and proliferating VS cells was determined as before. As previously shown,[27] trastuzumab (100 μg/mL) and PD158780 (20 μmol/L) each significantly reduced VS proliferation ($P < .05$) in cultures receiving sham IR (Fig. 4). Neither inhibitor caused a significant further reduction in cell proliferation in cultures treated with 30 Gy or 40 Gy ($P > .05$), indicating a lack of additive benefit of ErbB2 inhibition with higher doses of IR in reducing cell proliferation.

With regards to modifying the apoptotic response, PD158780, but not trastuzumab, significantly increased VS cell apoptosis ($P = .033$) in cultures receiving sham IR (Fig. 5). There was no additional apoptosis when PD158780 was combined with 30 Gy or 40 Gy IR. Trastuzumab significantly reduced the percent of apoptotic VS cells in response to 30 Gy and 40 Gy ($P < .05$), indicating
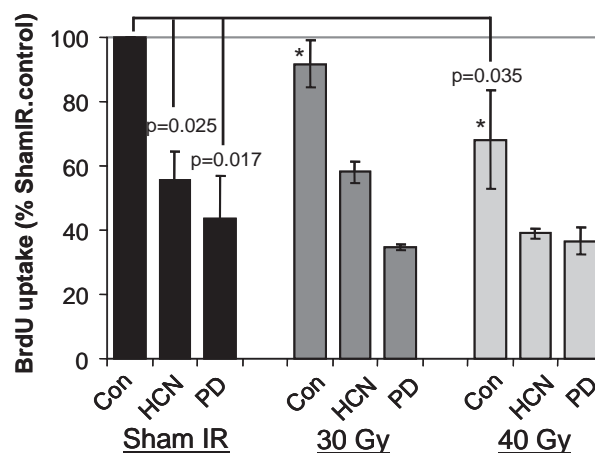

Fig. 4. Combination of irradiation (IR) with ErbB2 inhibitors provides no further decrease in vestibular schwannoma (VS) cell proliferation. Primary VS cultures were irradiated as above in the presence or absence (control, CON) of trastuzumab (Herceptin, HCN 100 μg/mL) or PD158780 (PD, 20 μmol/L). Control cultures received sham IR. BrdU uptake was determined as above. *P* values indicate significant differences by analysis of variance (ANOVA) with post hoc Hidak-Solm analysis. *Data also presented in Figure 4.
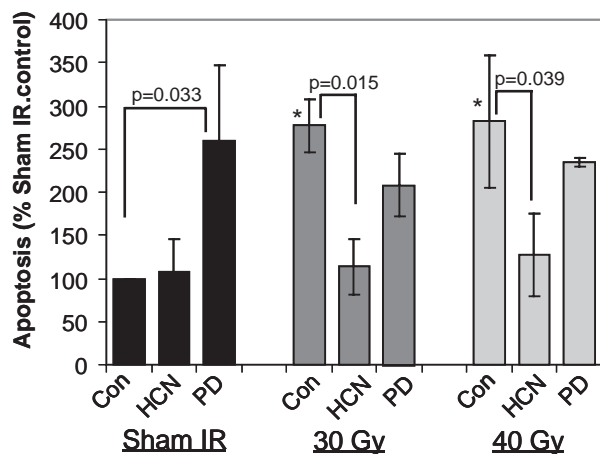
Fig. 5. ErbB2 inhibitors protect vestibular schwannoma (VS) cells from radiation-induced apoptosis. Primary VS cultures were irradiated as above in the presence or absence (control, CON) of trastuzumab (Herceptin, HCN 100 μg/mL) or PD158780 (PD, 20 μmol/L). Control cultures received sham IR. Apoptosis was determined as above with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). *P* values indicate significant differences by analysis of variance (ANOVA) with post hoc Hidak-Solm analysis. *Data also presented in Figure 2.
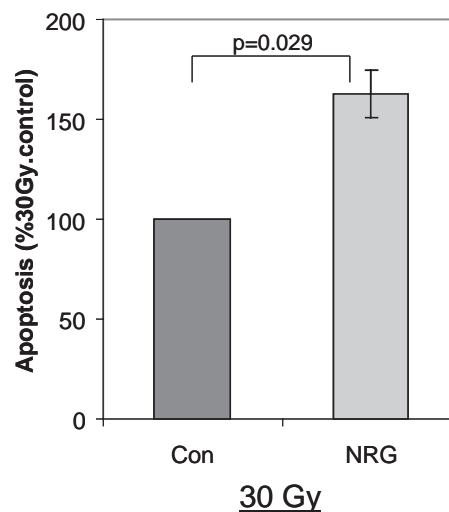
Fig. 6. Neuregulin-1 (NRG1) increases radiation-induced vestibular schwannoma (VS) cell apoptosis. Primary VS cultures were irradiated with 30 Gy as above in the presence or absence NRG1 (3 nM) and the percent of apoptotic VS cells was determined as before with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). NRG1 significantly increased the percent of VS apoptotic cells (*P* = .029) by Student *t* test.

a cytoprotective effect of the ErbB2 inhibitor (Fig. 5) and suggesting that constitutive ErbB2 signaling does not significantly contribute to the relative radioresistance of VS cells.

Vestibular Schwannoma Cell Radiosensivity Depends on Proliferation Rate

ErbB2 inhibitors decrease VS cell proliferation[27] (Fig. 4) and trastuzumab decreases VS cells' sensitivity to radiation-induced apoptosis (Fig. 5), suggesting that the apoptotic response of VS cells to IR depends on their proliferation status. To further test this possibility, we treated VS cultures with the ErbB2 ligand, NRG-1 (3 nM), a SC mitogen that we have previously shown to promote VS cell proliferation.[27] NRG-1 significantly increased the percent of apoptotic VS cells following 30 Gy IR (Fig. 6) (*P* = .029, Student *t* test) indicating that increased ErbB2 signaling enhances VS cell sensitivity to IR, likely by promoting mitosis. Taken together these results imply that the apoptotic response of VS cells to IR depends on their proliferative capacity. ErbB2 activation by exogenous NRG-1 promotes proliferation and radiation-induced apoptosis, while ErbB2 inhibition reduces proliferation and radiation-induced apoptosis.

DISCUSSION

Effects of Irradiation on Vestibular Schwannoma Cells

An increasing number of VSs are being treated with SRS/FSR;[5] however, the effects of IR on the VS cells themselves are not well understood. Here we evaluated the apoptotic and proliferative response of cultured primary VS cells to increasing doses of IR. Our data demonstrate that compared to most neoplastic conditions, VS cells in vitro are relatively radioresistant, requiring over 20Gy IR

(e.g., 30–40 Gy) to induce apoptosis and cell cycle arrest. Further, in doses up to 40 Gy, the reduction in proliferation is less than that seen with ErbB2 inhibition. These findings correlate well with those of Anniko,[45] who also noted that cultured VS cells respond only to high doses of IR. While it is difficult to extrapolate dose response relations from cultured cells to those for VSs in patients, these data are consistent with a lack of complete tumor regression in many VSs using current SRS/FSR protocols, and raise the possibility that the response of VSs to SRS/FSR may not be due to direct cytotoxic effects on the VS cells. Rather, the central necrosis seen on magnetic resonance imaging in some cases, and the reduced tumor growth in many cases may reflect indirect effects, for example, by decreasing tumor vascularity. Lee et al.[47] found viable, typical schwannoma cells in four VSs resected for growth following SRS and noted increased fibrosis of the tumor bed and surrounding tissues. In a separate study, the proliferation rate of 6 VSs resected for continued growth following SRS was less than the proliferation rate in 15 VSs resected for growth following incomplete microsurgical resection.[48] However, two irradiated VSs in that study showed markedly increased proliferation.[48] Thus, the effect of SRS/FSR on VS cell proliferation in vivo at the doses currently used clinically, whether direct or indirect, remains to be defined.

The relative radioresistance of VS cells to IR likely reflects their low proliferative capacity rather than an increased resistance to DNA damage.[41] We find that sublethal doses of IR rapidly induce double-stranded DNA breaks, evidenced by H2AX phosphorylation, in over 90% of VS cells. The potential for additional radiation-induced genetic aberrations leading to malignant transformation or secondary neoplasia highlights the need for long-term follow-up of patients managed with SRS/FSR. In some

Hansen et al.: ErbB2 Signaling and Irradiation of Vestibular Schwannoma Cells

cases, late growth correlated with malignant transformation.[8,49] Radiation-induced DNA damage holds particular implications for management of patients with NF2 who harbor germline *merlin* defects and are prone to radiation-induced additional genetic aberrations[8,50] and neoplasia.[7,51]

Modern treatment strategies of unilateral, sporadic VSs employing microsurgery or SRS/FSR are highly effective with limited morbidity. By contrast, schwannomas in patients with NF-2 present significant treatment challenges. They are less responsive to radiotherapy, more likely to recur following surgical removal, and carry higher morbidity with treatment.[35–37] Further, in NF2 patients with multiple, large schwannomas, surgical and SRS/FSR options become limited. Development of alternative therapies that improve or replace microsurgery or SRS/FSR would greatly benefit these patients.

One strategy to increase tumor responsiveness to IR is to provide radiosensitizing agents. Since most do not specifically target tumor cells, they have the disadvantage of also increasing the radiation-induced damage to non-tumor cells, raising the likelihood of side effects. An alternative strategy is to use reagents that specifically target tumor growth in combination with IR in an effort to achieve an additive response. Here we evaluated ErbB2 signaling, which provides a radioprotective effect for some carcinomas[40,46] as one potential target to enhance VS cell radiosensitivity.

Effect of ErbB2 Inhibitors on Vestibular Schwannomas Cells

Previous work has shown that ErbB2 is constitutively active in VS cells and that this contributes to their proliferative response,[27] and the data here confirm these findings since both trastuzumab and PD158780 reduced VS cell proliferation in these cultures. Several mechanisms possibly contribute to this constitutive ErbB2 signaling, including increased receptor expression, increased receptor trafficking to the cell membrane,[17] and autocrine NRG-1 ligand expression.[27]

In addition to promoting SC and VS cell proliferation, ErbB2 signaling promotes SC survival. We find that at the doses used in this study, PD158780, a pharmacologic ErbB2 inhibitor, induces VS cell apoptosis, but trastuzumab, a humanized ErbB2 inhibitory monoclonal antibody, did not. This difference may reflect more effective ErbB2 inhibition by PD158780 or non-specific effects of the pharmacologic compound such as inhibition of other tyrosine kinases (e.g., EGF receptor) required for VS cell survival. Thus, the role of ErbB2 signaling in VS cell survival will require further investigation with additional specific ErbB2 inhibitors.

Response of Vestibular Schwannomas Cells to γ-Irradiation Depends on Proliferation Status

Two observations from these studies demonstrate that the sensitivity of VS cells to IR depends on their proliferation rate. First, trastuzumab, which reduces VS cell proliferation, decreases the percent of apoptotic VS cells following lethal doses of IR. Second, NRG-1, a potent SC and VS cell mitogen,[27] increases the apoptotic re-

sponse to IR. In so far as these in vitro data can be extrapolated to the response of VSs in patients, they suggest that SRS/FSR would be more likely to induce apoptosis in growing VSs compared with static tumors. Further, concurrent treatment with ErbB2 inhibitors and SRS/FSR may reduce, rather than augment, the apoptotic response.

Despite our observation that the sensitivity of VS cells to IR depends on their proliferation rate, several reports indicate that VSs in patients with NF2, which presumably have an increased proliferative capacity, are more likely to grow following SRS/FSR than sporadic VSs.[35–37] Whether this reflects an underlying greater radioresistance of VSs from NF2 patients compared with sporadic VSs or is simply due to the greater growth potential of the remaining viable tumor cells requires further investigation.

CONCLUSIONS

With the rapidly increasing use of SRS/FSR to treat VSs, the effects of IR on VS cells require definition. We show that IR induces cultured VS cell apoptosis and cell cycle arrest in doses exceeding 20 Gy. Increasing VS cell proliferation with the ErbB2 ligand NRG1 enhances the apoptotic effect, while ErbB2 inhibitors, which reduce proliferation, protect VS cells from radiation-induced apoptosis. The relative radioresistance of VS cells likely reflects their low proliferative capacity and raises the possibility that the effects of SRS/FSR on VSs are predominantly indirect.

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